Experimental
ONCOLOGY

EFFECTS OF RADICAL OXYGEN SPECIES AND NO: FORMATION OF INTRACELLULAR HYPOXIA AND ACTIVATION OF MATRIX METALLOPROTEINASES IN TUMOR TISSUES

A.P. Burlaka, E.P. Sidorik, I.I. Ganusevich*, S.P. Osinsky
R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, Kiev, Ukraine

Aim: To establish the association between the radical oxygen species (ROS) and NO levels in the tumor cells mitochondria, between cell hypoxia development and activation of matrix metalloproteinases–2 and -9. *Materials and Methods:* Electron paramagnetic resonance (EPR) at room temperature and at the temperature of liquid nitrogen (77 ˙K), spin traps technology, enzymography in polyacrylamide gel were applied. *Results:* Redox-centers in the respiration cascade of mitochondria have been revealed, multiple oxidative damage of which in breast and liver cancer tissues of experimental animals as well as in tumor tissue from patients with gastric cancer promote the development of cell hypoxia. Involvement of ROS and NO in activation of latent forms of matrix metalloproteinases in gastric tumor tissues has been shown. *Conclusion:* We hypothesize that superoxide radical-anions participate in development of cell hypoxia in tumors and surrounding normal tissues inducing activation of latent forms of matrix metalloproteinases.
Key Words: carcinogenesis, cell hypoxia, radical oxygen species, nitric oxide, mitochondria, matrix metalloproteinases, electron paramagnetic resonance, spin trap.

In last few years the mechanisms of oxidative stress are in the focus of many investigators, mainly because of their role in etiology and pathogenesis of many diseases, in particular malignant tumors. Oxidative stress includes wide spectrum of different processes associated with each other, such as increased cell production of superoxide radical-anions ($O_2^{\cdot-}$), nitric oxide (NO), down regulation of functional efficiency of enzymatic and non enzymatic ROS detoxication systems, and oxidative damage of molecular structures of the cells. Different agents can trigger oxidative stress: inherited and induced genetic abnormalities (mutations), chemical carcinogens, ionizing radiation, stochastic events which include fluctuation of metabolic pathways and the level of cell redox-components.

Most oxidative metabolic pathways, redox-transferase and active centers, that are able to single electron reduction of the oxygen with superoxide radical-anions production, are localized in mitochondria [1–5]. Except ROS, mitochondria are a source of radical forms of NO (RFNO). NO is synthesized by mitochondrial form of NO synthases (mNOS) and/or as result of nitrite (NO_2^-) reduction, depended on status of electron transferring mitochondrial chain [6, 7].

From our point of view, the data on ROS activate expression of some matrix metalloproteinases (MMP) in epithelial and connective tissues are of special value [8–10].

It has been shown, that ROS could provide such activation through transcription factors that regulate MMP expression [8]. MMP is a family of Zn-containing endopeptidases, which have ability to destroy components of the extracellular matrix (collagen, gelatin, elastin) and are important for physiological processes, linked with tissue reconstruction such as embryogenesis, morphogenesis, angiogenesis, reparation [8, 11]. It is well known, that tumor cells and connective tissues induce synthesis of the enzymes (MMP is one of them), which eliminate physical barriers by proteolysis of extracellular matrix macromolecules that is obligatory condition for invasion and metastasis [11, 12].

Today we know nearly 20 types of MMP, that differ by substrate specificity and are divided on 5 subgroups: collagenases, stromelysins, gelatinases, membrane binding MMPs and the group of poorly studied MMPs [8, 11, 12]. Most investigators study the group of gelatinases, that decompose gelatin (type IV collagen), basic structural protein of basement membranes. These include so called gelatinases A and B, or MMP-2 and MMP-9 respectively. Now there are the data on up-regulated activity of these enzymes at different pathological states, including tumor progression [8, 13–15].

Basic control of total MMPs activity consists the regulation of their synthesis, activation and the suppression of their activity by specific tissue inhibitors (TIMP). MMP synthesis on transcription level is regulated by cytokines, hormones, growth factors, oncogenes, products of extracellular matrix degradation. Then MMP secretion appears in cell in latent proenzyme form (proMMP). Activation of the proenzyme, according to the current point of view, can be performed via few different pathways. It is well known that membrane bound MMP and TIMP play important role in this process [8, 11–13].

The main goals of our investigation were: to determine redox-centers in mitochondria respiration cascade, which are sensitive to ROS and RFNO damage, and to establish correlation between this damage and the rate of the ROS and RFNO production in tumor and normal tissues during chemical carcinogenesis in rats and in human gastric cancer; to study mechanisms of

MMP-2 and MMP-9 latent forms activation in gastric tumors and in normal surrounding tissues.

MATERIALS AND METHODS

Subjects of investigation. In total, 94 rats, bred in vivarium of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kiev, Ukraine), and fed with chow diet, were used. All animal experiments were carried out according to the rules of local Ethic committee. To induce the development of breast adenocarcinoma, female rats were treated with 7,12-DMBA, and to induce hepatomas, male rats were treated with NDEA according to [6]. Postoperated material from 48 patients with primary gastric cancer ($T_2N_0M_0$, differentiation stage G2), have been investigated [12]; these tissue samples include tumor tissue, normal surrounding tissue (all layers of gastric wall) and peripheral blood samples used for isolation of neutrophils.

EPR assay. The rate of superoxide radical-anions generation was assessed in tissue and blood neutrophils by using EPR and 2,2,6,6-tetramethyl-4-oxypiperidin spin trap at room temperature in special quartz cuvette. NO level was determined by diethylditiocarbamide spin trap (Sigma, USA). Level of RFNO in complex with non-haem Fe-S proteins in electron transferring chains of mitochondria membrane was revealed at temperature of liquid nitrogen (77 °K) [6].

Determination of MMP-2 and MMP-9 latent forms activation by superoxide radical-anions and RFNO. Taking into account that neutrophils may generate ROS and RFNO [17], cell suspension prepared from tumor tissue and control tissue (1 g of fresh tissue) was incubated with blood neutrophils (1000 cells) in sodium phosphate buffer (pH 7.4) at 37 °C for 12 h, then centrifuged 5 min at 3500 rpm. MMP activity was determined in the supernatant, RFO and RFNO levels were assessed in pellet.

Concentration of MMP-2 and MMP-9 latent and active forms were determined by enzymography in 12% polyacrylamide gel with SDS and 0.1% of gelatin as substrate [18]. Electrophoresis was carried out at +4 °C, 150 V for 4 h. After protein separation, gel was washed in 2.5% Triton X-100 solution, then incubated in buffer with $CaCl_2$ (pH 7.5) for 18 h at 37 °C, fixed and stained with 0.25% Coumassie brilliant blue and visualized as described earlier [18]. Estimation of proteolytic activity was done by measurement of the area of lysis zone, using standard kit for MMP-2 and MMP-9 (Sigma, USA). Results were evaluated using standard program TotalLab1.01.

Statistical analysis was performed using computer programs Statistics 6.0 and Excel 2003. For comparison of variables, mean ± standard deviation, Student's *t*-criterium, unifactorial dispersal analysis (ANOVA) were used.

RESULTS AND DISCUSSION

We have analized EPR spectra of experimental breast adenocarcinoma and hepatoma and gastric tumor tissues. Triplet structures with g = 2.007 in EPR spectra (Fig. 1–3) reflect the degree of mitochondrial

membrane electron-transfering chain damage caused by RFNO. Intensity of the signal alteration in EPR spectra of tumor tissues correlates with the level of NO production. The level of NO synthesized by mNOS in liver tissue samples (29 weeks after administration of carcinogen, when microscopic hepatomas were firstly visualized), reached 4.2 ± 0.05 nM/g of fresh tissue (Fig. 1). In hepatoma mitochondria (when tumor diameter was 1.5–2 cm), the level of RFNO was 5.3 ± 0.07 nM/g of fresh tissue, while for normal tissue this parameter was 1.5 ± 0.08 nM/g of fresh tissue (Fig. 1).
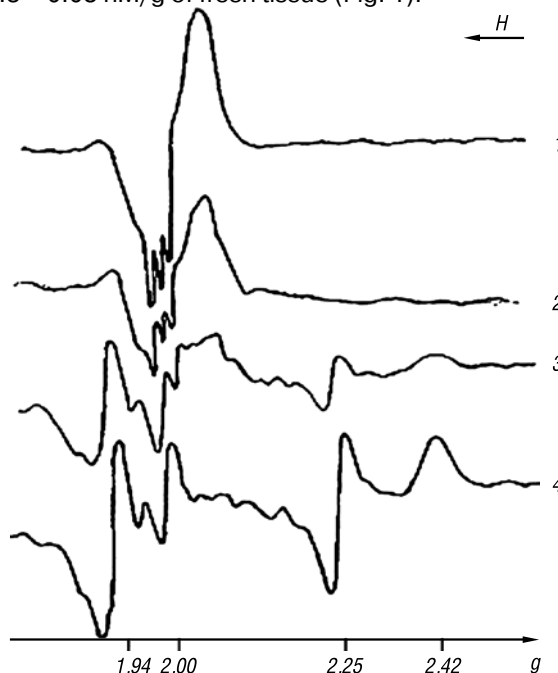
Fig. 1. EPR spectra of liver and hepatoma tissues upon tumor development. 1 — hepatoma (1–2 cm in the diameter); 2,3 — liver containing microcsopical hepatoma nodes; 4 — intact liver

Formation of nitrosil complexes at the different stages of hepatoma development (NO-Fe-S-proteins of mitochondria respiration chain), visualized as triplet structures in EPR spectra (see Fig. 1), induces the loss of functional activity by mitochondria through the damage of electron transporters. The electron loss occurs from the mitochondrial electron-transporting chain, process of four-electrons total reduction of the O_2 molecule to the H_2O disrupts. At these conditions, the initiation of the superoxide radical-anions in the mitochondrial respiration chain appears, the level of which significantly increases and 2 to 3 times exceeds the control value. At this stage efficiency of balance between oxidation and phosphorylation disrupts, that could be the cause of the development of cell hypoxia. We have detected the formation of the NO complexes with non-haem Fe-S-proteins in energetic system of breast adenocarcinoma cells at different stages of tumor development, NADH-ubichinonoxidoreductase and succinatedehydrogenase in particular, the level of which damage correlates with NO accumulation (Fig. 2). In tumors with diameter of 5 cm, NO level yielded 4.8 ± 0.09 nM/g of fresh tissue, and in tumors with size not more than 1.0 cm, it was 3.6 ± 0.04 nM/g of tissue. It has to be emphasized, that in lactating mammary

gland the level of NO was 1.65 ± 0.06 nM/g of fresh tissue. The rate of superoxide radical-anion generation in tumor specimens exceeds control values (see Fig. 2).
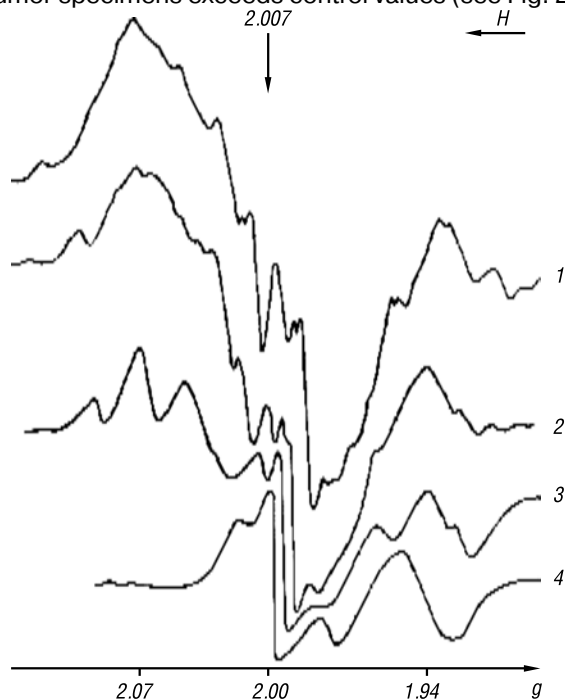
Fig. 2. EPR spectra of mammary carcinoma upon tumor development. 1 — mammary carcinoma (3–5 cm in the diameter); 2,3 — mammary carcinoma (0.5–0.8 cm in the diameter); 4 — lactating mammary gland

We have found similar damage of the components of mitochondrial electron-transporting chain in gastric tumors and surrounding normal tissues, caused by RFNO associated with Fe-S-proteins, and the intensity of such damage correlates with the level of NO synthesis in studied tissues (Fig. 3).

Fig. 3. EPR spectra of gastric cancer (1) and normal surrounding tissue (2)

The NO level in gastric tumors samples reached 5.5 ± 0.02 nM/g of fresh tissue, while in normal gastric tissues NO level was 0.24 ± 0.05 nM/g (see Fig. 3). The rate of superoxide radical-anion generation in gastric tumors and in normal gastric tissues was 1.8 nM/g of fresh tissue per min and 0.41 nM/g of fresh tissue per min respectively.

So, interaction of NO with the components of mitochondrial electron-transporting chain could to be critical for the formation of cell hypoxia, since the damage of NADH- ubichinonoxidoreductase and succinatedehydrogenase occurs, leading to incomplete reduction of oxygen in the process of oxidative phosphorylation, increase of the oxidative and NO stresses. In the case of NADH-ubichinonoxidoreductase, NO interacts with its components in the presence of low molecular weight S-nitrozothiols or peroxynitrite, which are able to transnitrozation or oxidation of the thiol components in this site of respiration chain. In succinatdehydrogenase, NO binds to its Fe-S-clusters, impairing electron transport in this branch of the chain.

NO can reacts with reduced forms of ubichinone, with the formation of NO^- and ubisemichinon radical. This reaction occurs at low rate in those branches of mitochondrial electron-transporting chain, where increased NO concentration appears, leading to formation of peroxynitrite [19]. NO-initiated inhibition of the cytochrom c oxidase activates generation the super oxide radical-anion, which is the source of the peroxinitrite formation in this branch of respiration chain, initiating secondary damage of the redox-centers and the increase of cell hypoxia [20–23].

Analyzing our data, we can conclude, that down-regulation of electron transfer in mitochondrial respiratory chain, recalled by interaction of NO radicals with its components, induces superoxide radical-anions generation, interrupts the balance between the processes of oxydation and phosphorylation and promotes cell hypoxia.

Taking into account the data, that the level of cell hypoxia correlates with MMP-2 and MMP-9 activities in tumor cells [24], we have investigated the effect of the radical forms of oxygen and NO on the activity of these proteases in gastric cancer tissue and normal surrounding tissues.

It was found out, that during the incubation of gastric tumor cell suspension with neutrophils from the same patients, the level of $O_2^{\cdot-}$ generation was 2.56 nM/$5.7 \cdot 10^3$ cells/min, the level of MMP-2 activity was 190 ng/g of fresh tissue, pro-MMP-2 – 290 ng/g of fresh tissue, and the same parameters for MMP-9 were 310 ng/g of fresh tissue and 360 ng/g of fresh tissue, respectively. Simultaneously, the levels of MMP-2 and pro-MMP-2 activity in this tissues after their incubation with neutrophils, which generated radicals of oxygen with the rate of 5.2 nM/$5.7 \cdot 10^3$ cells/min, were 400 ng/g of fresh tissue and 80 ng/g of fresh tissue, respectively. At the same conditions, the level of MMP-9 was 600 ng/g of fresh tissue, and pro-MMP-9 – 70 ng/g of fresh tissue. Otherwise, high levels of superoxide radical-anions generation

correlated with the increase of MMP-2 and MMP-9 expression (Fig. 4).
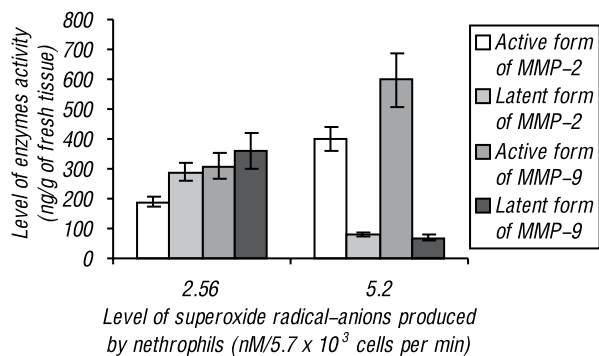
Fig. 4. Activities of MMP-2 and MMP-9 in gastric cancer tissue after incubation with neuthrophils of peripheral blood

We have established a direct positive correlation between MMP-2 and MMP-9 activities and the level of NO, produced by neutrophils from the patients with gastric cancer. So, when the level of NO in incubation media was $2.55 nM/5.3 \cdot 10^6$ cells, the levels of MMP-2 and pro-MMP-2 were 180 ng/g of fresh tissue and 300 ng/g of fresh tissue, respectively. Indices of MMP-9 and pro-MMP-9 activities were 320 ng/g of fresh tissue and 160 ng/g of fresh tissue, respectively. Whilst the NO level was $4.51 nM/5.3 \cdot 10^6$ cells, MMP-2 activity was 490 ng/g of fresh tissue, and that of pro-MMP-2 – 50 ng/g of fresh tissue. MMP-9 activaty reached a value of 450 ng/g of fresh tissue (Fig. 5).
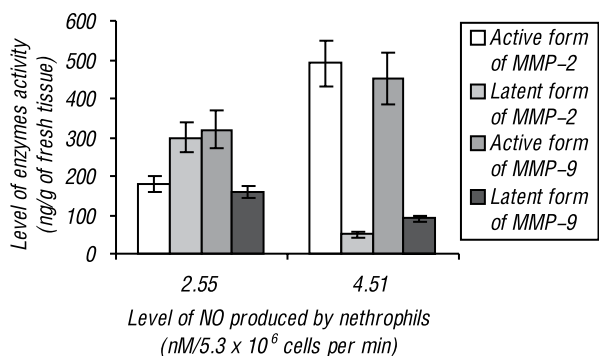
Fig. 5. Activities of MMP-2 and MMP-9 in gastric cancer tissue after incubation with neuthrophils of peripheral blood.

In the normal surrounding tissues, we have observed the same alterations of MMP-2 and MMP-9 activities, but they were less pronounced than those in tumor tissues.

The obtained results allow suggest that high levels of superoxide radical-anions and NO are able to activate latent forms of MMP-2 and MMP-9. Discussing possible mechanisms of such activation, we should note that latent forms of MMP-2 and MMP-9 contain pro-domain, carrying conservative sequence in active center that includes a residue of Cys and Zn^{2+}. Activation of pro-enzyme occurs by dissociation of Zn^{2+}-Cys bond [18]. We suppose that the break of Zn^{2+}-Cys-bond occurs via interaction with superoxide radical-anion and/or radical forms of NO, that serve as thiol-modifying agents, thus activating the latent form of enzyme.

REFERENCES

1. **Kushnareva Y, Murphy AN, Andreyev A.** Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. Biochem J 2002; **368**: 545–53.

2. **Turrens JF.** Superoxide production by the mitochondrial respiratory chain. Biosci Rep 1997; **17**: 3–8.

3. **Liang-Jun Yan, Rodney L, Levine E, Sohal RS.** Oxidative damage during aging targets mitochondrial aconitase. Proc Natl Acad Sci U S A 1997; **94**: 11168–72.

4. **Lenaz G.** The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology. IUBMB Life 2001; **52**: 159–64.

5. **Trumpower BL**. The protonmotive Q cycle. J Biol Chem 1990; **265**: 11409–12.

6. **Burlaka AP, Danko MI, Sidorik EP.** Kinetic patterns of the rate of generation and content of oxygen radicals in membranes of endoplasmic reticulum upon chemical carcinogenesis of liver and mammary gland. Dop Acad Sci Ukr 1994; **10**: 141–5 (In Ukrainian).

7. **Elfering SL, Haynes VL, Traaseth NJ, Ettl A, Giulivi C.** Aspects, mechanism, and biological relevance of mitochondrial protein nitration sustained by mitochondrial nitric oxide synthase. Am J Physiol Heart Circ Physiol 2004; **285**: 22–9.

8. **Davidson B, Reich R, Risberg B, Nesland JM.** The biological role and regulation of matrix metalloproteinases (MMP) in cancer. Arch Pathol 2002; **3**: 47–53.

9. **Kumar DA, Raju KV, Settu K, Kumanan K, Puvanakrishnan R.** Effect of a derivatized tetrapeptide from lactoferrin on nitric oxide mediated matrix metalloproteinase-2 production by synovial fibroblasts in collagen-induced arthritis in rats. Peptides 2005; **5**: 42–9.

10. **Egger B, Aubert JD.** Pulmonary emphyzema: mechanisms and therapeutic perspectives. Rev Med Suisse 2005; **16**: 2665–72.

11. **Curran S, Murray GI.** Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. Eur J Cancer 2000; **36**: 1621–30.

12. **Engers R, Gabbert HE.** Mechanisms of tumor metastasis: cell biological aspects and clinical implications. J Cancer Res Clin Oncol 2000; **126**: 682–92.

13. **Vihinen P, Kahari V-M.** Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. Int J Cancer 2002; **99**: 157–66.

14. **Chan LW, Moses MA, Goley E, Sproull M, Muanza T, Coleman CN, Figg WD, Albert PS, Menard C, Camphausen K.** Urinary VEGF and MMP levels as predictive markers of 1-year progression-free survival in cancer patients treated with radiation therapy: a longitudinal study of protein kinetics throughout tumor progression and therapy. J Clin Oncol 2004; **22**: 499–506.

15. **Kato K, Hara A, Kuno T, Kitaori N, Huilan Z, Mori H, Toida M, Shibata T.** Matrix metalloproteinases 2 and 9 in oral squamous cell carcinomas: manifestation and localization of their activity. J Cancer Res Clin Oncol 2005; **131**: 340–6.

16. **Panasenko OM, Chekanov AV, Arnold Yu, Sergienko VI, Osipov AN, Vladimirov YuA.** Generation of free radicals upon degradation of hydroperoxide in the presence of myeoloperoxydase or activated neutrophils. Biochemistry 2005; **70**: 1209–17 (In Russian).

17. **Nedelijkovic ZS, Gekce M, Loscalzo J.** Mechanisms of oxidative stress and vascular dysfunction. Postgraduate Med J 2005; **79**: 195–200.

18. **De Clerck YA, Perez N, Shimada H, Boone TC, Langley RE, Taylor SM.** Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer Res 1992; **52**: 701–8.

19. **Moncada S, Higgs A.** The L-arginine-nitric oxide pathway. N Engl J Med 1993; **329**: 2002–12.

20. **Sharpe MA, Cooper CE.** Interaction of peroxynitrite with mitochondrial cytochrome oxidase. Catalytic production of nitric oxide and irreversible inhibition of enzyme activity. J Biol Chem 1998; **273**: 30961–72.

21. **Brown GC.** Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. Biochim Biophys Acta 2001; **1504**: 46–57.

22. **Torres J, Sharpe MA, Rosquist A, Cooper CE, Wilson M.** Cytochrome c oxidase rapidly metabolises nitric oxide to nitrite. FEBS Lett 2000; **475**: 243–66.

23. **Radi R, Denicola A, Alvlrez B, Ferrer-Sueta G, Rubbo H.** The biological chemistry of peroxynitrite. USA Acad Press 2000; 57–82.

24. **Osinsky S, Ganusevich I, Bubnovskaya L, Valkovskaya N, Kovelskaya A, Sergienko T, Zimina S.** Hypoxia level and matrix metalloproteinases-2 and −9 activity in Lewis lung carcinoma: correlation with metastasis. Exp Oncol 2005; **27**: 202–5.

ЭФФЕКТЫ РАДИКАЛЬНЫХ ФОРМ КИСЛОРОДА И ОКСИДА АЗОТА: ФОРМИРОВАНИЕ ВНУТРИКЛЕТОЧНОЙ ГИПОКСИИ И АКТИВАЦИЯ МАТРИКСНЫХ МЕТАЛЛОПРОТЕИНАЗ В ОПУХОЛЕВОЙ ТКАНИ

Цель: установить взаимосвязь между уровнями образования радикальных форм кислорода и оксида азота в митохондриях клеток опухолевых тканей, развитием клеточной гипоксии и активностью матриксных металлопротеиназ − 2 и −9. *Методы:* электронный парамагнитный резонанс при комнатной температуре и температуре жидкого азота (77 °K), технология Spin Traps, зимография в полиакриламидном геле. *Результаты:* выявлены редокс-центры в дыхательной цепи митохондрий, множественные окислительные повреждения, которые при канцерогенезе в молочных железах, печени экспериментальных животных и в опухолевой ткани больных раком желудка способствуют развитию клеточной гипоксии; показано участие супероксидных радикал-анионов и оксида азота в активации латентных форм матриксных металлопротеиназ в ткани рака желудка. *Выводы:* высказано предположение, что в формировании клеточной гипоксии в опухоли и прилегающих нормальных тканях принимают участие супероксидные радикал-анионы, которые активируют при этом латентные формы матриксных металлопротеиназ.
Ключевые слова: канцерогенез, клеточная гипоксия, радикальные формы кислорода, оксид азота, митохондрии, матриксные металлопротеиназы, электронный парамагнитный резонанс.